



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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In re Application of:
Westbrook

Serial No.: 07/784,222

Filed: October 28, 1991

For: METHODS AND COMPOSITIONS
FOR THE DETECTION OF
CHROMOSOMAL ABERRATIONS

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Examiner: J. Fredman

Group Art Unit: 1634

Atty. Dkt: ARCD-010/NAK

CERTIFICATE OF MAILING 37 C.F.R. 1.8	
I hereby certify that this correspondence is being deposited with the U.S. Postal Service as First Class Mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on the date below:	
Sept 1, 1998	
Date	Richard A. Nakashima

DECLARATION UNDER RULE 131

I, CAROL A. WESTBROOK, HEREBY DECLARE AS FOLLOWS:

1. I am the named inventor of the subject matter claimed in the referenced U.S. patent application, Serial No. 07/784,222, filed October 28, 1991.

2. I understand that the Patent and Trademark Office Examiner in charge of examining this application has cited against my application the following publication:

Tkachuk *et al.*, "Detection of *bcr-abl* Fusion in Chronic Myelogeneous Leukemia by in Situ Hybridization," *Science* 250: 559-562, 1990.

3. The invention of claims 1-3 and 5-35 was made and tested in the United States prior to October 26, 1990, and therefore prior to publication of the cited article by Tkachuk *et al.*

4. The fact that the invention of claims 1-3 and 5-35 was made and tested in this country prior to October 26, 1990 is evidenced by studies set forth in the attached notebook extracts (Exhibit A). Among other things, this Exhibit sets forth the following studies which exemplify the practice of my invention:

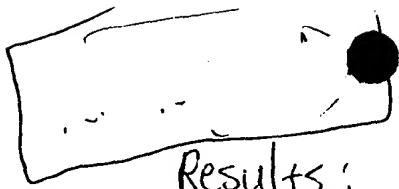
- a) Possession and use of the c-Hu-ABL, PEM12 and MSB-1 probes in *in situ* hybridization experiments for detection of chromosomal aberrations in leukemic cell lines and in blood cells from patients with leukemia (Pages 1-2 and 5-43 of Exhibit A).
- b) Identification of doublets in the chromosomal DNA of leukemic cell lines and blood cells from patients with leukemia using distinguishably labeled probes specific for the c-H-*abl* and *bcr* genes. (Pages 1-2, 5-6 and 21-24 of Exhibit A).
- c) A detailed protocol for detection of the c-H-*abl/bcr* fusion gene, using distinguishably labeled probes specific for the c-H-*abl* and *bcr* genes. (Pages 5, 7-11, 13-22, 24, 26-31, 33-43 of Exhibit A).

Each of items a) through c) as represented in the attached Exhibit were carried out in this country prior to October 26, 1990.

5. All statements made in this Declaration of my own knowledge are true and all statements made in this Declaration on information and belief are believed to be true, and these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both under 18 U.S.C. §1001 and may jeopardize the validity of this application or any patent issuing thereon.

Aug 3 '98
Date

Carol A. Westbrook
Carol A. Westbrook



Results:



① MH5.15 on ALH:

Cells "fuzzy" - lot of cytoplasm
no signal detected.

* Problem is slides? Too much cyto,
poor penetration of probe despite
Proteinase Treatment.

- will discard slides which look like this one -
make new control slides.

② Abl alone on SupB13

PERFECT! Clean slides, consistent signal
most c. 2 dots/nuclei.

③ Msh-1 alone on SupB13

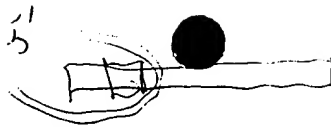
fair results - many nuclei c consistent
signal but background high

④ Double hybs:

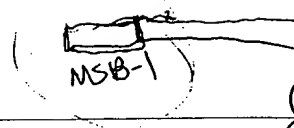
SupB13 - good results - clear consistent signal
but problem of timing -

Red signals seen first, fade quickly
as they fade green signals come up
Many doublets clearly visualized: red-green side by side.
Red background still high.

ALH - many double signals, no doublets
slide less satisfactory than other (SupB13)



2



PEB (+)

P1902

⊕ on PEGE

⊖ on SO. BCR

Conclusions

- Amplification step works & difficulty, & no obvious cross reactivity.
- MSB - shows good specific hyd'z
- Doublets obvious
- Need the computer imager! or More amplification to get photos

Mon: Will prepare new slides:

- controls
- Sup B13
- patient slides - have 3 patients
- ⊖ ALL from CALGB study

Work out steps / order reagents for another round of amplification

Make 10% BSA solns to mix for blocking
10% SSA

Mon

- Fluorescence + imaging box from library...
- Flow cytometry paper - ? nuclear suspension preparation
- Dropped 4 sets of slides:

JK control slides -
 J Smith - peripheral blood ALL
 P Orzell - bone marrow ALL
 Davis - bone marrow, frozen ALL

20-25 slides each, still some cells in fix 4°.
Rare mature PMNs or bands, but present in
 Smith + Orzell slides good quality
 Davis - poor cell "quality" Freezing artifact
 apparent.

- Thawed cells from Varga, Anne ALL Ph^+ , bcr^+
 to test 50×10^6 cells
 Incubated $\frac{1}{2}$ o/n
 " $\frac{1}{2}$ 3° to allow cells to
 recover from freezing

over

- Dr Grieme & Susan McKibben - senior student
5-5723

Discussed objective of my analyses at length -
trial run Thurs. AM.

Weds: Worked on Grant application...

Thurs AM: Dr. Grieme & Susan McKibben 5-5723

Took 319-scope down to lab -
images captured fuzzy, had problems w focus
used double-hyb-d SUPB13 from

Also, amount of light camera is sensitive too may be
too much for the fluorescent assay?
too much "junk"?

Uncertain, but worth pursuing at least until set-up
for fluorescence is perfected ~ then decide.

Thurs PM -

Harvested cells from Tues. - from Varga-PB
Good yield - did metaphase harvest.

Thoms PM

Hybridize Patient Varga: MSB-1, cHu-abl, + double
SuPB13 as control - double

① Ppt of DNA:

	Exp ⁺ DNA		Plac DNA	SSDNA	KOAc	ETOH	Dextr/Form
	MSB	ABL					
MSB ^{bst.}	✓ ₁₅	-	1	1	2	200	5/5
Abl ^{bst.}	-	✓ ₅	1	1	1	100	5/5
MSB/ABL ^{bst.}	✓ ₃₀	✓ ₁₀	2	2	5	500	10/10

Used "new" MSB-1

Abl

- almost gone

Into -70° x 30° - spin, dry, reconst.

5' at +70° - into 37°

② Slide prep.

- ✓① Slides on warmer - 1 1/2"
- ✓② RNase 1° 37°
- ✓③ Wash x 4 2xSSC 2"
- ✓④ Dehydrate & dry
- ✓⑤ Formamide 70% 70° 2'
- ✓⑥ Dehydrate & dry
- ✓⑦ Proteinase K 7.5' 37°
- ✓⑧ Dehydrate & dry
- ✓⑨ Hybridize - 90° x 2'
- ✓⑩ 37° O/N.

Results :

Specific signals seen on all, \bar{c}
many doublets

High background

CAN suggests the following :

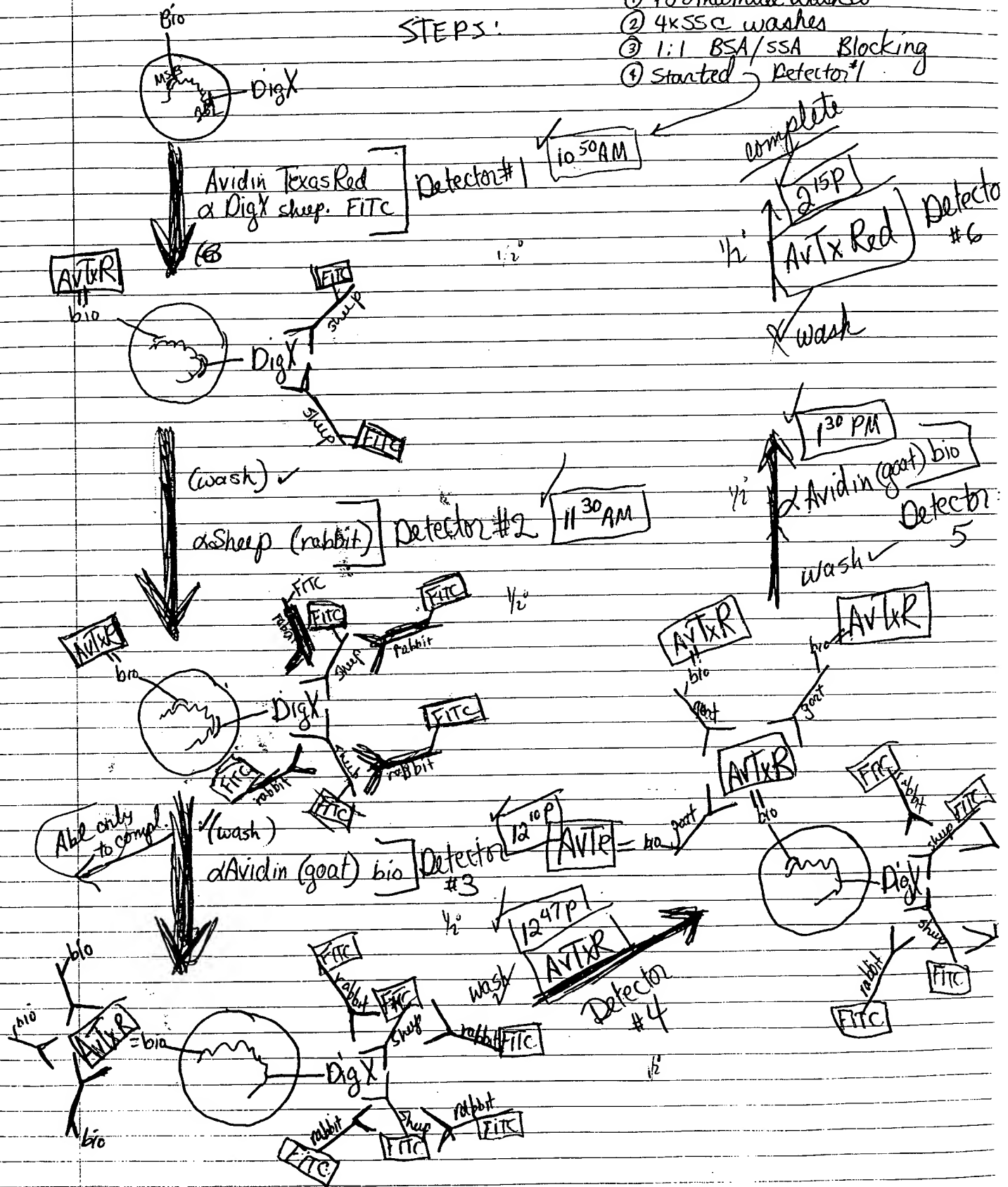
① Try \bar{c} one color only; technical aspects of 2-color
too much for most labs

②

FRIDAY: Detection Day ...

STEPS:

- ① Formamide washes
- ② 4xSSC washes
- ③ 1:1 BSA/SSA Blocking
- ④ Started Detector 1



Detector #1: Avidin Tx Red
 α DigX] in Sheep alb.
 3%
 for 2 slides: 600 -

✓ 400 μ l 4x/Triton
 ✓ 200 μ l 5% SSA
 20 μ l AvTxR
 20 μ l α DigX

For one Slide each:

Avidin TxR - see below
 α DigX < ~~400~~ μ l :
 375 μ l

✓ 256
 400 μ l 4x/Triton
 200 μ l 5% SSA
 ✓ 25
 10 μ l α DigX Fluorescein

Avidin TxR for: MSB-only slide 200
 Det. # 4 3 slides ~~800~~ 600
 # 6 3 slides ~~800~~ 600
 + 200
 1600 μ l
 \downarrow
 1800

✓ 1200 μ l 4x/Triton
 ✓ 600 μ l 5% SSA
 70 μ l AvTxR

α Avidin - bio (goat) AASB Detector #3 3 slides 600
 Detector #5 3 slides 600
~~200~~
 1400 \rightarrow 1500

✓ 1000 4x/Triton
 ✓ 500 5% SSA
 60 μ l Ab

α Sheep (rabbit) Detector #2 3 slides 600 μ l
 \downarrow
 800

✓ 800 μ l 4x/Triton
 ✓ 300 μ l ~~B~~
 30 μ l Ab

16
 20



DigX-label probes: PEM12, MSB1, KK5.33 (cosmid 5)

* Nick translate kits "pilfered". No Stop Buffer, no reaction buffer left, vials were mixed between the two kits...

DNase dil -

1:10 - 1 in 9
1:100 - 1^{1:10} in 9 of
1:500 - 2^{1:100} in 8

will try as no new kit available → ordered then

	Exptl DNA ^{1 µg}	10xRB	dNTPs	DigX-dUTP	water	1:500 DNase Dil
MSB1	✓4 ^{use 8} _{next time}	5	5	5	23	4
PEM12	7.5 ^{use 4} _{next time}	5	5	5	19.5	4
KK5.33	✓10 ^{use 4} _{next time}	5	5	5	17	4

Combined - 14° x 2°

Made 200mM EDTA: 20 µl 500mM EDTA
30 µl H₂O

5 x " " + 65° x 10'

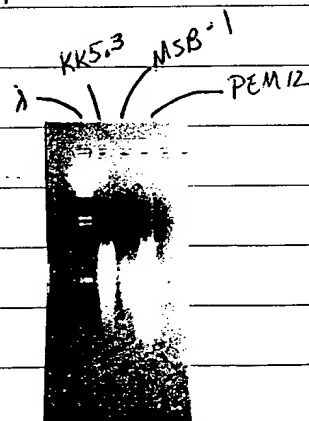
Ran out on gel:

KK5.33 + MSB1

need more cutting; PEM12 OK

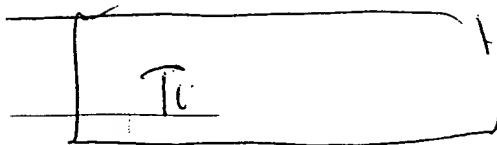
(? MSB concentration less)

↓ PEM12 more than ~~that~~
calculated by O.D. readings?



For AM:

- Will add MgAc (2 x 1M) to MSB + KK5.33 tubes & re-run them tomorrow when I nick-translate more MSB-1 + PEM-12 & bio-dUTP.
- Test DNase of new kit - old one running out.
- Order 2 new nick-trans. kits + HIDE THEM! (Enzo LK)
- " more digoxigenin-dUTP (Boehringer Mannheim 1093 08)
- order meshes for soft tissue work. \$100 for 25 nm



- I Re-do nick transl. of MSB + KK5.33
- II Test DNase dil. of new nick transl. kits
- III Ppt DNA for a hybr. run
- IV. Hybr. 6 slides - single color.
- V. Make more RNase

IV. Re-start nick-trans. for MSB-1 + KK5.33 - digX
New dilution 1:500 DNase -

1 λ into 9 RB = 1:10

1 1:10 into 9 DB = 1:100

2 into 8 = 1:500 ✓

Add 4 λ DNase

4 λ DNA Pol.

2 λ 1M Mg Ac

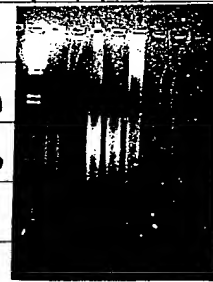
to each tube

Will ✓ p 1°

* out of DNA pol. from BOTH
KITS!

~~Probably cause of p~~

KK5.33 MSB
1:500 1:800 1:1000



KK5.33:
not OK
MSB-?
little DNA
New DNase
1:500 a

II. Test dilutions for new DNase : 1:500, 1:800, 1:1000

1:10 = 1 in 9 - 1 of 1:10 into 9 = 1:100

1 of 1:100 into 4 = 1:500

1 of " into 7 = 1:800

1 " " into 9 = 1:1000

	Control DNA	RB	TTP	dNTP	H ₂ O	DNase	DNA pol
1:500	✓4	✓5	5	✓5	13	4	4
1:800	✓4	✓5	5	✓5	13	4	4
1:1000	✓4	✓5	5	✓5	13	4	4

Stop p 2°

III. Make more stock RNase \rightarrow 10 5ml aliquots.

IV. Test digk-labeled probes from
PEM 12 + ~~MSB~~ MSB-1

adhol do double-labeling & single color

Sup B13 ~~MSB~~ MSB-1

2. MSB-1 / PEM 12

- 2 slides

3. MSB-1 / ~~PEM 12~~ / ABL

- 2 slides

BV 173

1. PEM-12

2. MSB-1 / PEM-12

3. MSB-1 / PEM 12 / ABL

~~MSB~~ ~~MSB~~ ~~MSB~~

	MSB	^{DNA} PEM	ABL	PlacDNA	ssDNA	KoAc	ETOH	Dextr. Form.
MSB-1 (7 slides)	15	-	-	1	1	2	200	5/5
PEM-12 (7 slides)	-	15	-	1	1	2	200	5/5
MSB/PEM (11 slides)	15	15	-	1	1	3	300	10/10
MSB/PEM/ABL (11 slides)	15	15	50	1	1	4	400	10/10

Combined - freez - ppt - reconst

① Slides on warmer

② into RNase 1°

③ 4x wash 2x SSC 2'

④ Dehydrate & dry : 3'

⑤ 70° formol 70° 2'

⑥ Dehydrate & dry

⑦ Proteinase K 7.5'

⑧ Dehydrate & dry

⑨ Hybridize & seal

90° 2' \rightarrow 37° O/N

Nick translation results:

* PEM 12 OK but a lot
of DNA - (will begin hyb's
c 10 λ instead of 15 λ)

* MSB-1 very little - next
nick trans. will double a.

* KK5.33 still didn't cut well

* DNase dil for 2nd kit at least

1:500

Weds:

✓ Make 4xSSC / 0.1% Triton
 ✓ Make 50% formamide / 4xSSC
 Make 4xSSC

→ into water bath

Lab meeting!

Dev. Slides:—

• one round amplification.

No major problems.

Thurs. — Guest lecturer

• ✓ worked on proposal

FR

New nick translation kits arrived:

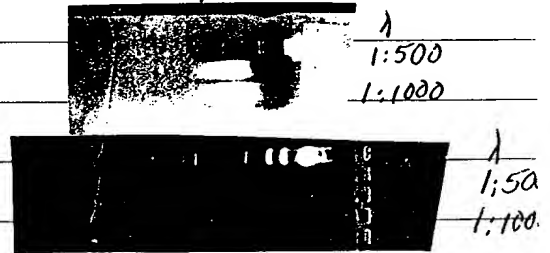
Taped one shut, & my name + into freezer. (Label)
 Other one "A" - will test DNase dilutions

1:500, 1:1000 + 1:1200
 1 in 9 → 1:10 → 1 in 9 = 1:100
 1 in 4 = 1:500
 1 in 9 = 1:1000
 1 in 11 = 1:1200

	Rxn Buff	dNTP	TTP	↓	Control	DNase	H ₂ O	DNase	DNA P _i
1:500	✓5	✓5	✓4	✓4	✓4	✓24	4	4	4
1:1000	✓5	✓5	✓4	✓4	✓4	✓24	4	4	4

added Mg Ac to rxn as our DNA
 is dissolving in TE - may be part of
 problem & past Nick-trans's.
 Didn't add enough at 1st, so

2^o of rxn, ran gel & some of reaction mix, added more Mg⁺⁺
 Mon - to rest, ran x1^o more.



Will use 1:500:

Nick translate MSB-1 & digX - will use more this
 PEM 12 & digX
 ABL & biotin

	Exp. DNA	MgOAc	Rxn Buffer	bio-dNTP	digX	dNTP	H ₂ O	1:500 DNase	DN
MSB	✓6 λ	2	✓5	0	✓5	✓5	190	4	4
PEM	✓7 λ	2	✓5	0	✓5	✓5	2018	4	4
ABL	✓5 λ	2	✓5	0	✓5	✓5	20	4	4
new MMS- MMS-50	✓2 λ	2	✓5	0	✓5	✓5	23	4	4

over.

Dilution DNase

1:10 1 in 9

61 in 9 = 1:100

4 in 165 = 1:500

2⁴⁵ - 4⁴⁵ Reaction Run.

5 λ Stop buffer 10' heating block

Will run gel in early AM. + gene-clean

~~Made reagents~~

In AM:

① Hybridize patient slides - direct preps from ALL, Katsikas -

using probes nick-translated today if they are OK on gel.

② Make reagents - 4xSSC, 2xSSC, @ 4xSSC/0.1% Triton

③ Prepare MSB-1 phage DNA for shipping @ rate.

③ Cells from Marina? and name of Houston doc (8p kbps)

Worked on Am. Ca Society Grant Proposal

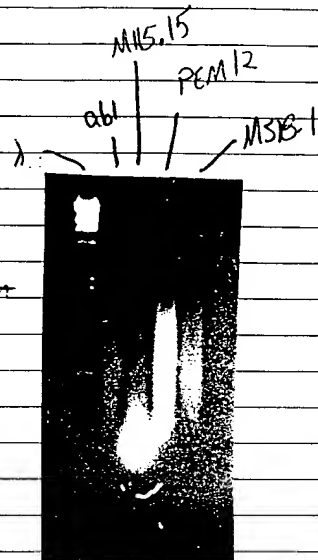
Turned in / Fed Ex'd FRI 5PM.

Range of probes nick-translated
last week.

Only MH5.15 did well, a lot of DNA used \rightarrow about right

MSB-1: ~~Still light~~ on the DNA (not enough) but better
PEM12 still heavy (too much)

Problem c Abl - ? don't know



Plan: 1. Restart rxns of Abl, MSB, PEM12
2. Concurrently run New ones - will need lots of probe for ALL study
still need digx - PEM12, MSB

For the "re start"

	MgAC ^{250mM}	DNA	dNTP	digx	bioUTP	1:500 DNase	DNA pol
Abl	1	1 λ	2	2	2	4	4
PEM12	1	1 λ	2	2	2	4	4
MSB-1	1	1 λ	2	2	2	4	4
total vol ~ 54 μ							

Need to add MgAC to 5mM in 50 μ

would be 1 μ of a 250mM solution

$$250 \text{ mM} = (0.250 \text{ M})(15 \text{ ml}) = (1 \text{ M MgAC}) \text{ stock} \times x$$

$$3.75 \text{ ml} = x$$

3.75 ml MgAC
11.25 ml H₂O

ON HOLD per CAW
will test them on the cells

For the "fresh start" in AM

	Exp DNA	Rxn Buff	dNTP	^{250mM} MgAC	bioUTP	digx	H ₂ O	1:500 DNase	DNA pol
Abl	7 λ	5	5	1	5	0	19	4	4
MSB-1	5 λ	5	5	1	0	5	21	4	4
PEM12	5 λ	5	5	1	0	5	21	4	4

Need 24 μ of DNase dil
17 μ of DNase

1 in 4

2 in 5

4 in 16

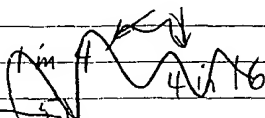
~~6 in 24~~

8 in 3

DNase dilution

1 in 9 = 1:10

1 of 1:10 in 9 = 1:100



5 of 1:100 into 20

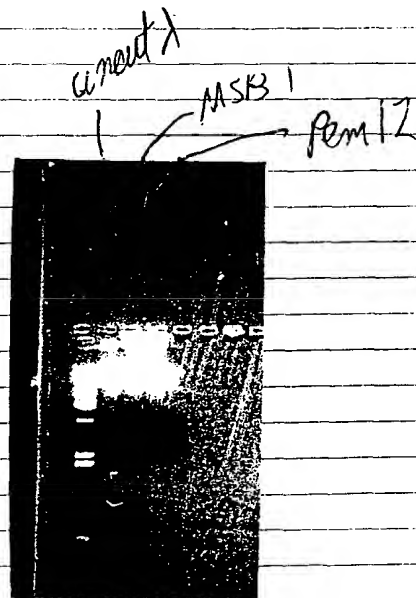
~~Start on~~

CAW suggests withholding
DNA pol. until DNase is done —
will try to run in AM

In the meantime: Will run gel (0.8%) to ✓ concentration/purity
of 8/2/90 phage prep.
with both HindIII cut $\phi\lambda$
and uncut $\phi\lambda$

3 λ PEM 126 λ MSB1 mg uncut $\phi\lambda$ 1 mg HindIII $\phi\lambda$

Conclusion:



	Exp. DNA	Rxn B.	dNTP	bioUTP	Dig V	H ₂ O	1:500 DNAse
Abi	✓5	✓5	✓5	✓5	✓0	22	4
MSB-1	✓6	✓5	✓5	-	✓5	21	4
PEM-12	✓3	✓5	✓5	-	✓5	24	4

↳ not enough. use 4x

Reaction started: 9³³ AM. → 14° Ran 1° 10'

Will Add 4x DNA pol. at 10⁴³ A.

Added DNA pol at 11⁰² A, ran 1°, heated to 65° x 10'
returned to 14° x 1 more hour.

Added 5x 200 mM EDTA. to stop.

DNAse dil: 1 in 9 = 1:10

1 of 1:10 in 9 = 1:100

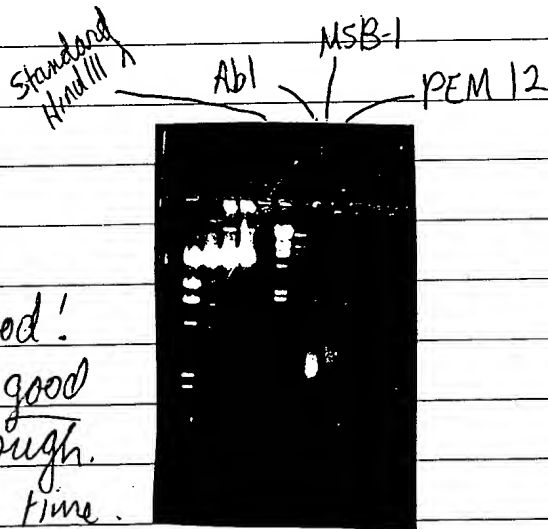
3 in 12 = 1:500 for 3 tubes

Ran on minigel to
✓ for cutting:

Conclusions: ① cutting good!

② Amt of MSB-1 (6x) is good
of PEM-12 (3x) not enough.

Will use 4x next time.



For Thurs: (Weds. is Fannie May/Coleman Cancer Conf.)

- ① hybridize six normal slides, comparing the probes from nick translation to those of today.
- ② get frozen tumor from Lori, try collagenase digestion.

LJ



I. Hybridize 6 nl slides to compare probes ~ 50000 c probes < 500

		Exp'l DNA	Plac DNA	ssDNA	KoAc	E+OH	Formd/DexSulf.
Abl	10/1	5	1	1	1	100	5/5
Abl	10/2	5	1	1	1	100	5/5
MSB-1	10/1	15	1	1	1.5	200	5/5
MSB-1	10/2	15	1	1	1.5	200	5/5
PEM12	10/1	15	1	1	1.5	200	5/5
PEM12	10/2	15	1	1	1.5	200	5/5

Precipitated DNA for each as above.

Six ALH 8/13 slides thru day #1 protocol;
fresh reagents, no problems.

II. Tumor digestion:

- Collagenase diluted to 2000 u/ml, aliquots of 1 ml frozen (used 10 mg of collagenase)
- Tumor # 90- from Loric, c - 5g + - 18g
- 4.5 ml media + minced tumor + 0.5 ml collagenase
- O/n incubation.

For AM:

- ① Develop slides, c one amplification step
- ② ✓ colon tumor digestion

I. Colon tumor digestion

Transferred cells + media + collagenase
to tube — allowed lg fragments to settle
~~Spun~~ ^{Removed} cells in suspension, returned large
pieces of tissue to flask + more collagenase

Spun suspended cells — ? hopefully single cells
Hypotonic KCL + fix 3:1
Dropped a test slide → Bacteria! No ep. cells

This approach won't work — ? fix first then digest
Need to find new protocol

II. Detection steps for hybridized slides:

50% formamide x3, 4xSSC x3, serum block x1', then:

A. For biotin-Abl slides

Avidin Texas Red in 2.5% BSA / 4% Triton X 200/400
amplified + —

10 μ biotin- α -Avidin (in goat) in: 400 4xSSC/Triton X
200 3% SSA

+ another round of Avidin Texas Red as above

B. For DigX-MSB1 + DigX-PEM-12

15 μ α digX (in sheep) in 800 4xSSC/Triton X
400 3% SSA

then amplify:

FITC- α sheep (in rabbit) in 800
400

Washed 3' x2 in 4xSSC / 1% Triton X between 1' +
amplification steps.

Results -

All slides show signal: bright, clear, specific

PEM12

both excellent

MSB-1

= OK but usable

MSB-1

better

Abl-

OK but

Slides hazy - Problem c̄ BSA?

background high in FITC/DigX slides

- may need to add rabbit serum to blocking step

- Wash x3 in. between 1° Ab + amplification step

Conclusions:

- ① All probes usable. Size, ^{difference} seems to be important for MSB-1 only, & then only a little
- ② Need work on Ig background

FOR NEXT WEEK:

- ① Hybridize patient slides
- ② " CML colony cells from Toronto
- ③ Try again c̄ another disaggregation protocol

	PEM12	MSB-1	ABL	PlacDNA	ssDNA	KOAC	EtOH	Form/des
PEM/AbI x5	60λ	-	25λ	5	3	9	300	25/25
MSB/AbI x3	45λ	45λ	15λ	3	2	6.5	200	15/15
MSB 15/slide								
PEM 12/slide								
PPT + reconstitute DNA.								

Slides to be done: (8)

BV 173 : Control for PEM-12 AbI x1
 Toronto CML Slides : PEM12-AbI x2
 JSmith : ALL-PB (unknown) : " " x1
 SUPB13 control for MSB-AbI x1
 JSmith ALL-PB (unknown) MSBAbI x1
 Varga ALL-PB (known) MSB-AbI x1
 Varga ALL-PB (known) PEM12-AbI x1

5 PEM12-AbI
 3 MSB-AbI

- ✓ ① Slide warm: (55° X 4')
- ✓ ② RNase 37° X 1'
- ✓ ③ 2x SSC x4 2'
- ✓ ④ Dehydrate + dry
- ✓ ⑤ 70% formamide / 4xSSC x2' 70°
- ✓ ⑥ Dehydrate + dry
- ✓ ⑦ Proteinase K
- ✓ ⑧ Dehydrate + dry
- ✓ ⑨ Hybridize + seal
- ✓ ⑩ 90° x2', 37° o/n

For tomorrow :- Slide detection steps

- Prepare journal club
- Talk to Tony Montag re: slides (if any);
 at lunch to discuss tissue and

I. Detection steps

✓ 1. Formamide 50% wash x3 5'

✓ 2. 4xSSC washes

✓ 3. Blocking 5% BSA/SSA/3% Rabbit 10A

✓ 4. Detector #1 (see 14 Sept 90)

AvTxRed) for 8 slides : 1600 + 200 for filter = 1800
 Biotin)
 1200 4xSSC/Trit
 600 SSA

~10 μ l/ml

20 μ l λ AVTxR
 20 μ l λ α DigX

✓ wash x3

✓ 5. Detector #2

 α sheep in rabbit serum :

1200 4xSSC/Trit
 600 ~~sheep~~ Rabbit serum
 20 μ l α Sheep

✓ wash x3

✓ 6. Detector #3

bio- α Avidin (goat) in sheep

1200 4x/Trit
 600 SSA
 20 μ l α AV

✓ wash x3

✓ 7. Detector #4 Avidin Tex Red

1200 4xSSC/Trit
 600 SSA
 20 μ l AV Tx R

wash x3

~~8. DAPI x3'~~

9. Wash 4xSSC x1

10. DAPCO/coverslip

Will examine slides in AM,
 do counts
 take photos.

cont d

Cells were intact, unlike Varga who had been frozen. Preparation fairly clean, moderate background.

3. Positive control slides. SupB13-MSB/ABL had very yellow nuclear background staining, poor hybridization and no doublets seen. Bad prep for unclear reasons. But the high yellow nuclear background is similar to the first successful run - may be a property of the cell line, or may need different treatment. Will think it over!

BV173-PEM/ABL with good hybridization, little background, multiple clear doublets in the majority of cells.

"RULES OF THE ROAD" FOR INTERPRETING AND COUNTING PREPS:

1. Count only intact nuclei with clearly visible rim. Skip smudged, partly smashed, or fractured nuclei.
2. Count only nuclei which have instantly obvious red and green signals. If one of the colors is missing from the first sight of the nucleus, skip it.
3. Score only obvious doublets. If inconclusive, look carefully for other single signals in other parts of nucleus. If still not sure and no single signals seen, skip it. If other single signals seen, count as "no doublet".
4. Do not count in areas of high background or debris on the slide, or in areas where nuclei are clumped and obscure the individual nuclear borders.

PROBLEMS WITH COUNTING:

1. Can only count one or two nuclei in a field at a time before the red fades, so in fields with lots of nuclei, only a few get counted. May circumvent this somewhat by partially closing down the first diaphragm along the tube, just outside the lamp housing.
2. Difficult sometimes to define what is a doublet: how close must be a translocation and how close by chance? More than the diameter of the signal away? Not a constant number! Need to hybridize and count some normals (oops, no negative controls!).
3. Signals in slightly different planes of focus means will miss several if not constantly playing with fine focus.
4. Tiring! Need to set up scope on a more comfortable table!

Took 32 photos, ASA 800, will develop at 800. Exposure times were around 7-10 seconds, tried to expose until the red faded.

* Still need more amplification, more blocking, and better washing.

Thurs -

Not in Lab - ACS luncheon

Fri.

Dropped pt slides

Made touch preps of Colon case 90-11690

- tumor
- adenoma
- nl mucosa

2 slides of each fixed in
95% ~~ethanol~~ ^{ethanol} + small amt
glacial acetic acid
rest in 95% alcohol

M.

I. Hybridization

II. Colon tissue disaggr.

III. Microscope

IV. Call A. Keating

V. Drop rest of patient slides.

I. Hybridization - will do following cases:

1. Varga, 48° culture (dropped this AM) @ PEM/ABL
2. Varga 48° culture " MSB/ABL
to ✓ results @ better cells - last prep
was @ recently thawed cells

3. SupB13 @ MSB/ABL
 4. ALH normal @ MSB/ABL
 5. ALH normal @ PEM/ABL - neg control for double
- } pos & neg controls

Colon cells:

6. 90-11690 tumor touch prep 95% alc :
7. 90-11690 nl mucosa 95% alc : ? no ep. not hoped for this.
8. ALH normal lg's : control (in case # 7 is no good and control for probe.

Precipitation / reconst. of probes

DNA

	PEM	MSB	ABL	MHS.15	placDNA	ssDNA	KoAc	ETOH	dextr/fo
Pem(2) ABL	24	-	40	-	1	✓1	✓2	✓500	10/10
MSB(3) ABL	-	56	46	-	✓1.5	✓1	✓5	✓400	15/15
MHS.15(3)	-	-	-	✓80	✓1	✓1	✓2	✓500	15/15

(MSB+PEM: 12/sl.) (MHS.15 6/sl.) (Abl 5/sl.)

Combine thru ETOH, 1/2° @ -70°, spin, dry.

Reconstitute @ dextran/formamide + 70° x 5 min, then 37°.

Probes used: MSB - DigX

PEM12 - DigX

Abl - bio

MH 5.15

Steps:

- | | |
|-------------------------|-----------------------------------|
| ① Warmen 65" x 4" | ⑤ Dehydrate + dry |
| ② RNase x1" 37° | ⑥ Proteinase K 7.5' 37° |
| ③ Wash 2X SSC x4 2' | ⑦ Dehydrate + dry |
| ④ dehydrate + dry | ⑧ Hybridize + seal |
| ⑧ 70% formamide 70° x2' | ⑩ Denature 90° x2', O/N 37° → 3PM |

II. Colon tissue: acquired 1 more case colon tissue -

- ① 90-11726 (Gray, Arlene) Low ant. res - colon ca.

fungating centrally ulcerated polypoid tumor.

Touch preps made of tumor → into 95% alc. alone
Normal mucosa into MEM & pen/strep, refrigerated
several hours. Transferred to 4% paraformaldehyde at.

- ② 90-11690 ml mucosa fresh in fridge over weekend.

Cut off strips of mucosa, minced it as fine as possible
in MEM. Divided specimen in half:

a. 1/2 of cells: 4ml MEM + 0.5ml collagenase 200 u/ml,
into 25 ml flask: into incubator o/n.

b. 1/2 of cells: transferred to paraformaldehyde.
Will do a collagenase digestion & o/n fixation.

Paraformaldehyde prep per Manuelidis

1. 1.6 gm paraformaldehyde into 20 ml H₂O → heat 60" to
2. add equal vol. buffer - I used NaH₂PO₄ (pH 6), 0.2 M
3. adjusted pH & NaOH to 6.8 - 7.2 (6.93)

III. Microscope: Zeiss rep. not in office - will try tomorrow
IV. Told med. Dr. hinting at unsuccessful results on 1st try & CML colo

TUES. 14

I. Detection steps, yesterday's run.

II. Begin new hybridization run w/ PEM/ABL

III. Colon tumor disaggregation, cont'd.

I. Detection steps:

A. Remove coverslips, wash X3 50% formd. at 40 C.

B. Wash X3 for 3 min, 4X SSC, 40C.

C. Blocking: used 50/50 5% BSA/SSA.

D. Detection/amplification: see diagram page.

II. New hybridization run. Will use following slides:

1. CML colony slide W-150, Day 0, 7,8
2. CML colony slide W-150, Day 0, 9,10.
3. BV 173, pos. control.
4. ALH 8/13, neg. control.
5. Vargos, ALL BM
6. Lord, ALL PB
7. J Smith, ALL
8. Orzell, ALL

All to be done with Pem/ABL.

DNA preparation: Probes used, PEM-12-digX ?
ABL-biotin

- all used

	PEM12	ABL	placDNA	ssDNA	KOAC	EtOH	Dex/Form
#1	✓44	✓20	✓4	✓4	✓7	500	20/20
#2	✓44	✓20	✓4	✓4	✓7	500	20/20

Combine, -70 X30 min, spin, dry, reconstitute.

+70 X5 min, then 37 until use.

Slide prep:

- ✓1. Slide warmer 65 C X4 hr.
- ✓2. RNase X1 hr, 37 C.
- ✓3. Wash 2X SSC X4 2 min.
- ✓4. Dehydrate & dry.
- ✓5. 70% formd. 70 C X2min.
- ✓6. Dehydrate & dry.
- ✓7. Proteinase K 37 C 7.5 min.
- ✓8. Dehydrate & dry.
- ✓9. Apply probe & seal.
- ✓10. 90 C x2 min, then 37 C o/n.

all slides EXCEPT ALH neg control were on X4°

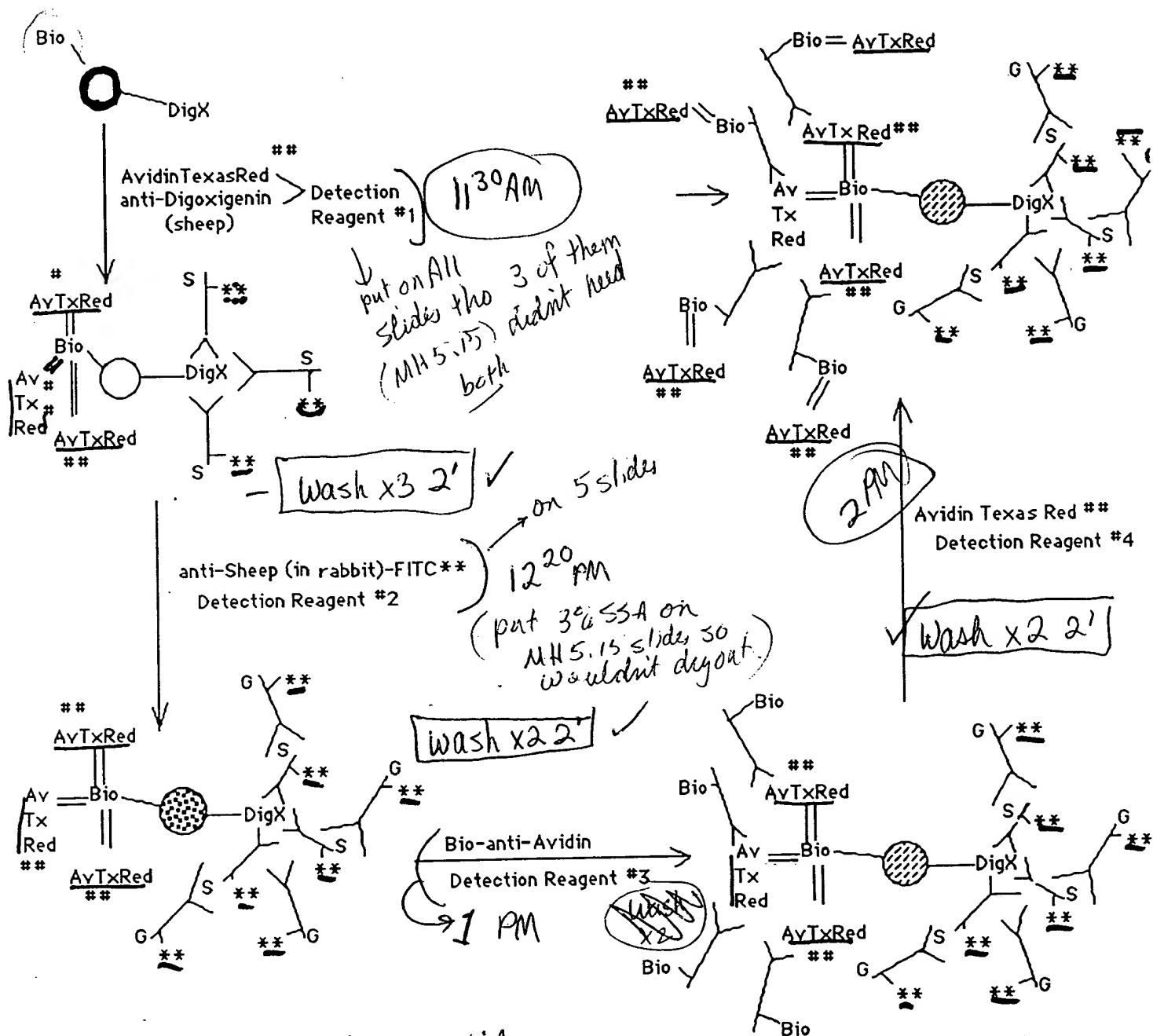
III. Colon tumor disaggregation:

cont'd incubation @ collagenase.

Will sieve in AM, pass into PBS, ? trypsinize?

In Situ Amplification Steps and Reagents

Yaremko



Detection reagent #1: 8 slides

✓ 1200 ml 4x SSC / 0.1% Triton

✓ 600 ml 3% SSA

✓ 20 μ l TxRAvidin

✓ 20 μ l α DigX

Detection reagent #2 for 8 slides

✓ 1200 ml 4x trit

✓ 600 ml 3% SSA

✓ 20 μ l α sheep-FITC. 5
 Do not put MH 5.15 slides

Detection reagent #3 for 8 slides

1200 ml 4x trit

600 ml 3% SSA

20 μ l Bio α Av.

over

Detection reagent #4

✓ 1200 x 4x/trit.

✓ 600 x 3% SSA

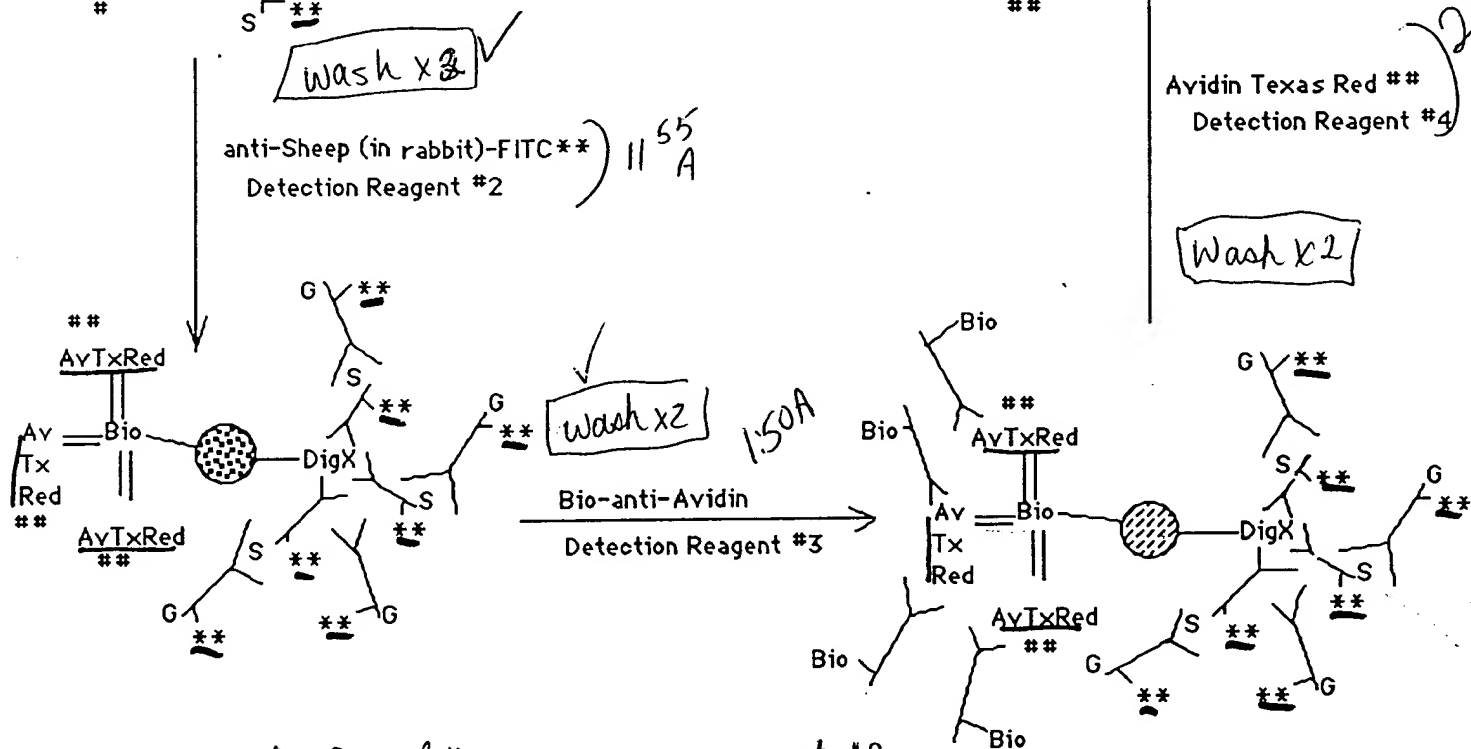
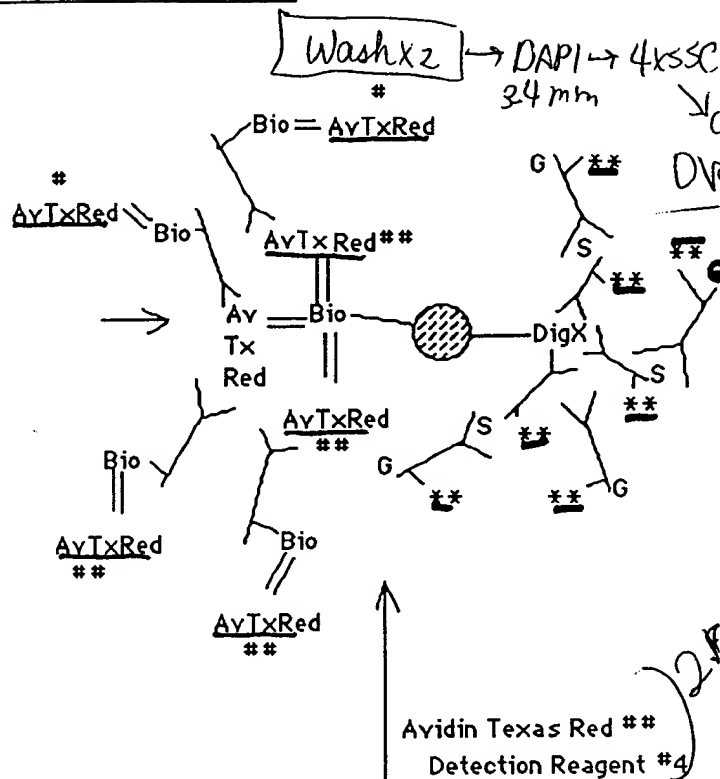
✓ 20 x AvTxR

Will examine + photograph Thurs. AM
 @ the results of Tomonow's cases.

For Weds

1. Detection steps on slides
2. Colon disaggr.
3. ? review heating slides?
4. ✓ for fax sheets from Zeiss rep.

1. formamide $\times 3$
2. 4xSSC $\times 3$
3. Block ~~SSA~~ 5% SSA ~~SSA~~ = 945 p.
Bio



Det. reagent #3
1200 4xSSC
600 SSA
20% α Avidin

Det reagent 4
1200 4xSSC
600 5SA
20% Av Tr Red

- Completed at 3PM.
- JSm PEM/SBL slide cracked when plotted mounted & taped it to a 2nd slide - may not be able to view it.

- 11415.

Colon tumor slides with not enough penetration of probes, and not enough single cells. Will try these again with stronger and longer proteinase K digestion.

will not do
today -
will do @
PEM/ABL run
Mon.

- 3-8 to be done with
MSB/ABL.

1, MSB-digX 10/1/90: 10 ul/slide
 ABL-biotin 10/1/90: 5ul/slide
~~PEM12-DigX 9/18/90: 10 ul/slide~~

lacDNA	ssDNA	KOAC	EtOH
4 6	4 6	7 10	500 300

dry, reconstitute.

30
ERROR

Put in 6
of 53 Dr
place
Too Much
Supp
2.

1. Slide warmer 65 C X4 hr.
2. RNase X1 hr, 37 C.
3. Wash 2X SSC X4 2 min.
4. Dehydrate & dry.
5. 70% formd. 70 C X2min.
6. Dehydrate & dry.
7. Proteinase K 37 C 7.5 min.
8. Dehydrate & dry.
9. Apply probe & seal.
10. 90 C x2 min, then 37 C o/n.

Not enough prob. to cont., so will do tomorrow

14

cont'd

III. Nick translate.				DigX-UTP or bio-UTP	H ₂ O	DNase	DNApol.
	<u>Exp.DNA</u>	<u>RxnB</u>	<u>dNTP</u>				
Abl	✓4	✓5	✓5	✓5 (bio)	✓23	✓4	4
MSB	✓6	✓5	✓5	✓5 (DigX)	✓21	✓4	4
Pem	4	5	5	5 (DigX)	23	4	4

DNase dilution 1 in 9 = 1:10

1 of 1:10 into 9 = 1:100

3 of 1:100 into 12 = 1:500.

Combined all but DNA pol. Reaction started: 2230.

DNA pol added at: 3:19 P.

Reaction stopped w/ 5 ul 200mM EDTA, 10' at 65 C.

Will run on minigel in A.M.

IV. Order supplies , 2 of each:

1. Sigma D5527 Dulbecco's PBS 500ml \$8.50
2. Sigma H 8389 CMF-Hank's BSS 500ml \$9.25
3. Vector: Rhodamine-600-Avidin D A-2005 \$50 5 mg.

Make: ✓4x SSC

✓4x SSC/0.1% Triton

5% Rabbit serum

5% SSA

✓RNAse soln.

In AM

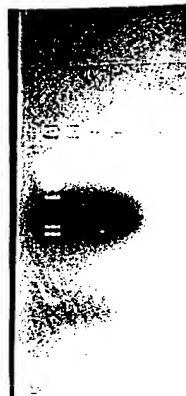
1. Screen all slides - discard all bad ones
keep good ones.

2. Run gel

3. complete hybr. if probes OK



I. Ran gel - first one A standard
 then ABL
 MSB
 PEM



No cutting at all!

So, did not complete ISH run.

Amounts, however, seem OK. (4, ABL) (6, MSB) (4, PEM) (2)

Will proceed as follows:

Mon: Another nick translation reaction -

1. DNase 1°
2. DNA pol + DNase 1°
3. DNA pol 1°

Run gel: if OK, gene clean + prepare for
 a run Mon. + Tues. with all slides.

II. Colon disaggreg. - working out steps of disaggr. from
 Jakoby + Pastan, cell culture, (see printouts)

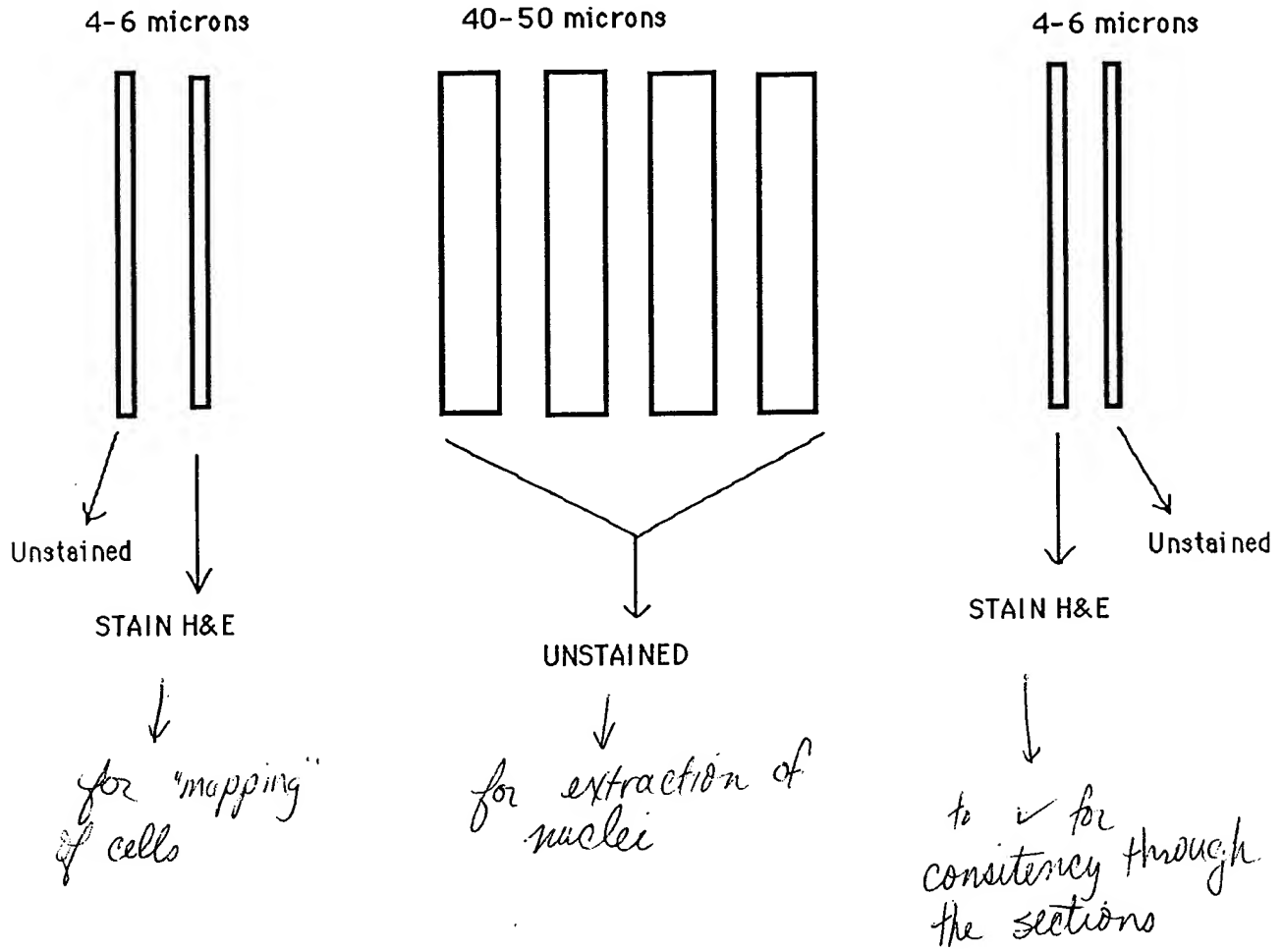
2) Asked T. Montag to have blocks cut from recent
 colon ca case ... as per accompanying diagram.

Will include these cells in runs @ fresh and
 briefly fixed tissue.

3) Materials ordered.

90-11690 COLON TUMOR AND ADENOMA

Blocks number: C, E, G.



Will save unstained slides - ? factor VIII stains to look for vessel in
 ? ~~tumor~~

LY

- I. Nick translate
- II. Make enzyme solutions and paraformaldehyde, Tris quencher
- III. Pick up slides from T. Montag
- IV. Acquire tissue from pathology.

I. Nick translate.

	<u>Exp.DNA</u>	<u>RxnB</u>	<u>dNTP</u>	<u>DigX-UTP</u> <u>or bio-UTP</u>	<u>H2O</u>	<u>DNase</u>	<u>DNAPol.</u>
Abl	4	5	5	5 (bio)	23	4	4
MSB	6	5	5	5 (DigX)	21	4	4
Pem	4	5	5	5 (DigX)	23	4	4

DNase dilution 1 in 9 = 1:10

1 of 1:10 into 9 = 1:100

3 of 1:100 into 12 = 1:500.

Combined all but DNA pol. Reaction started: 8:35 AM

DNA pol added at: 9³⁵ADNase stopped w/ 10' at 65 C at: 10³⁵ADNA pol. cont'd x1 hr, stopped w/ 5 ul 200mM EDTA at: 11³⁵A

Ran gel:



A - Abl - MSB - PEM12

all a good cutting.

labelled

Success! So repeated reaction with more Pem12 & MSB 1:

Nick translate.

	<u>Exp.DNA</u>	<u>RxnB</u>	<u>dNTP</u>	<u>DigX-dUTP</u>	<u>H2O</u>	<u>DNase</u>	<u>DNAPol.</u>
MSB	6	5	5	5 (DigX)	21	4	4
Pem	4	5	5	5 (DigX)	23	4	4

*(prepared 2 reaction tubes of each)

DNase dilution 1 in 9 = 1:10

1 of 1:10 into 9 = 1:100

4 of 1:100 into 16 = 1:500.

(cont'd)

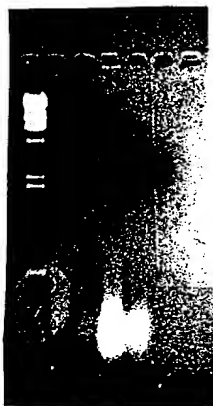
Combined all but DNA pol. Reaction started: 1:50 PM

DNA pol added at: 2:50 P

DNase stopped w/ 10' at 65 C at: 3:50 PM

DNA pol. cont'd x1 hr, stopped w/ 5 ul 200mM EDTA at: 4:50 PM

Ran gel:



All OK. will Gene Clean.
Amts?

PEM 12 into 120 +
100% of TE

MSB into 75% each

↓
combined, for
150% MSB

II.
Made 500 cc 0.1 M Tris pH 7.2
50 ml 1 M Tris + 450 H₂O
Autoclave in AM.

220% PEM

Labeled

10/25

Will make rest of enz solns in AM.
as well as 3+4.

Called Rush immunology, asked for copy
of disaggreg. protocols for paraffin sections

T:

- LY I. Hybridization run
 II. Paraffin tissue disaggregation protocol
 III. Prepare enzyme solutions

I. Hybridization run. Will use following slides:

- | | |
|---|----------------------|
| 1. CML colony slide W-150, Day 0, 11,12 | |
| 2. CML colony slide W-150, Day 0, 17,18 | 1-4 to be done with |
| 3. BV173, pos. control. | PEM/ABL. |
| 4. ALH 8/13, neg. control. | |
| 5. Vargas, ALL BM x2 | 9-10 to be done with |
| 6. Lord, ALL PB x2 | MSB/ABL. |
| 7. J Smith, ALL x2 | |
| 8. Orzell, ALL x2 | 5-8 w/ both. |
| 9. SUPB13, pos. control. | |
| 10. ALH neg. control. | |

Total of 14 slides. 8 for PEM/ABL, 6 for MSB/ABL.

DNA preparation: Probes used, MSB-digX 10/22/90: 12 ul/slide
 ABL-biotin 10/22/90: 5ul/slide
 PEM12-DigX 9/22/90: 12 ul/slide → *all used!*

	MSB1	PEM	ABL	placDNA	ssDNA	KOAC	EtOH	Dex/Form
P/A	--	94	40	8	8	15	500	40/40
M/A	72	-	30	8 8	8 8	11	500	30/30

Combine, -70 X30 min, spin, dry, reconstitute.
 +70 X5 min, then 37 until use.

Slide prep:

- | | |
|-----------------------------|---------------------------------|
| 1. Slide warmer 65 C X4 hr. | 6. Dehydrate & dry. |
| 2. RNase X1 hr, 37 C. | 7. Proteinase K 37 C 7.5 min. |
| 3. Wash 2X SSC X4 2 min. | 8. Dehydrate & dry. |
| 4. Dehydrate & dry. | 9. Apply probe & seal. |
| 5. 70% formd. 70 C X2min. | 10. 90 C x2 min, then 37 C o/n. |

II. Tissue disaggregation.

Over.

LY

Tissue Disagg.

Rec'd nl colon mucosa case 90-12079.
pt # 1956909

Divided & minced into

fresh 4% paraformald.
Fixed 2°

0.1 M TRIS x1°

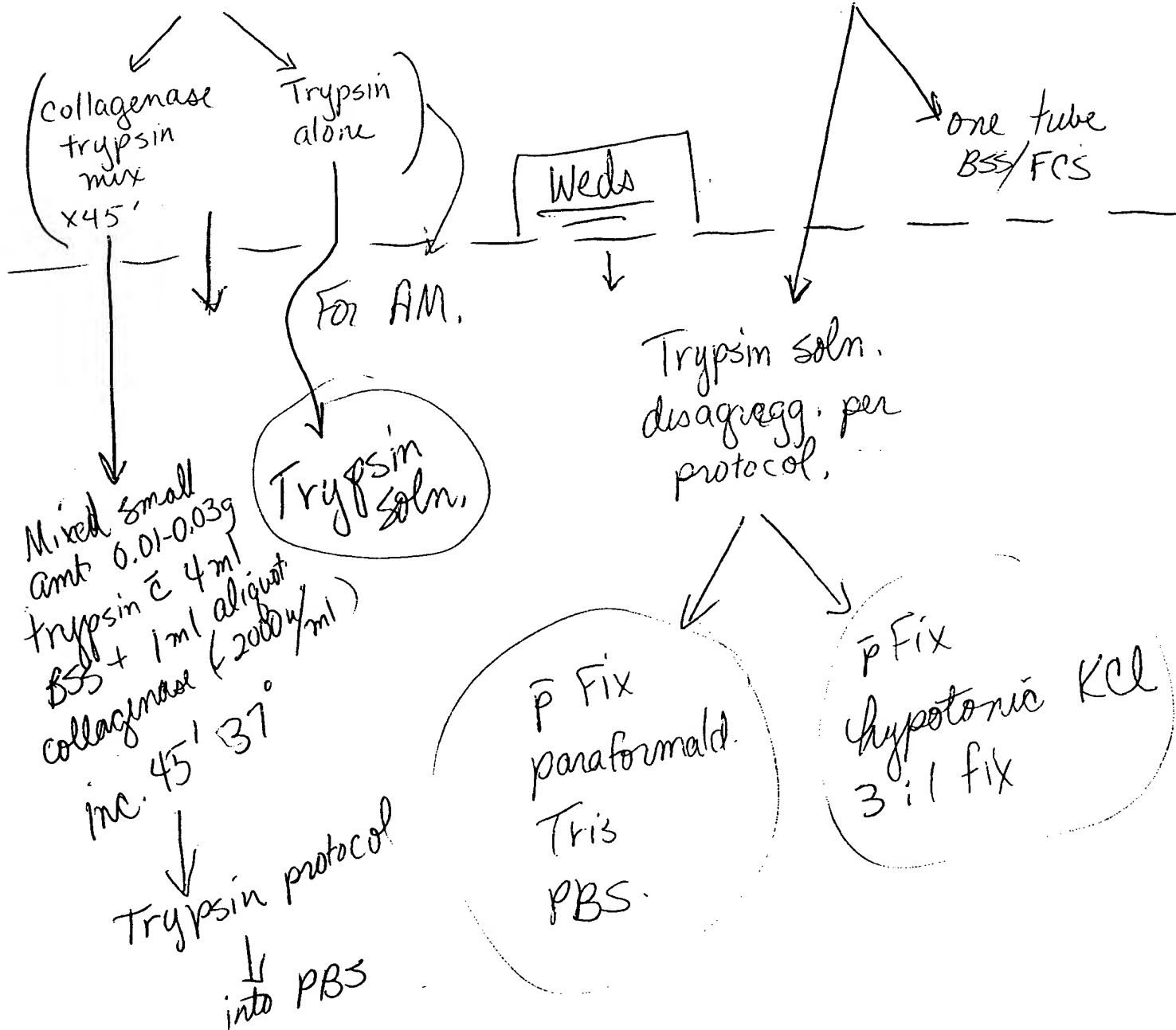
↓ spun

PBS c sodium
azide

CMF - BSS.
Kept at 37° while mixed & filtered
trypsin/co

↓

added double vol. FCS -
refrigerated o/n.



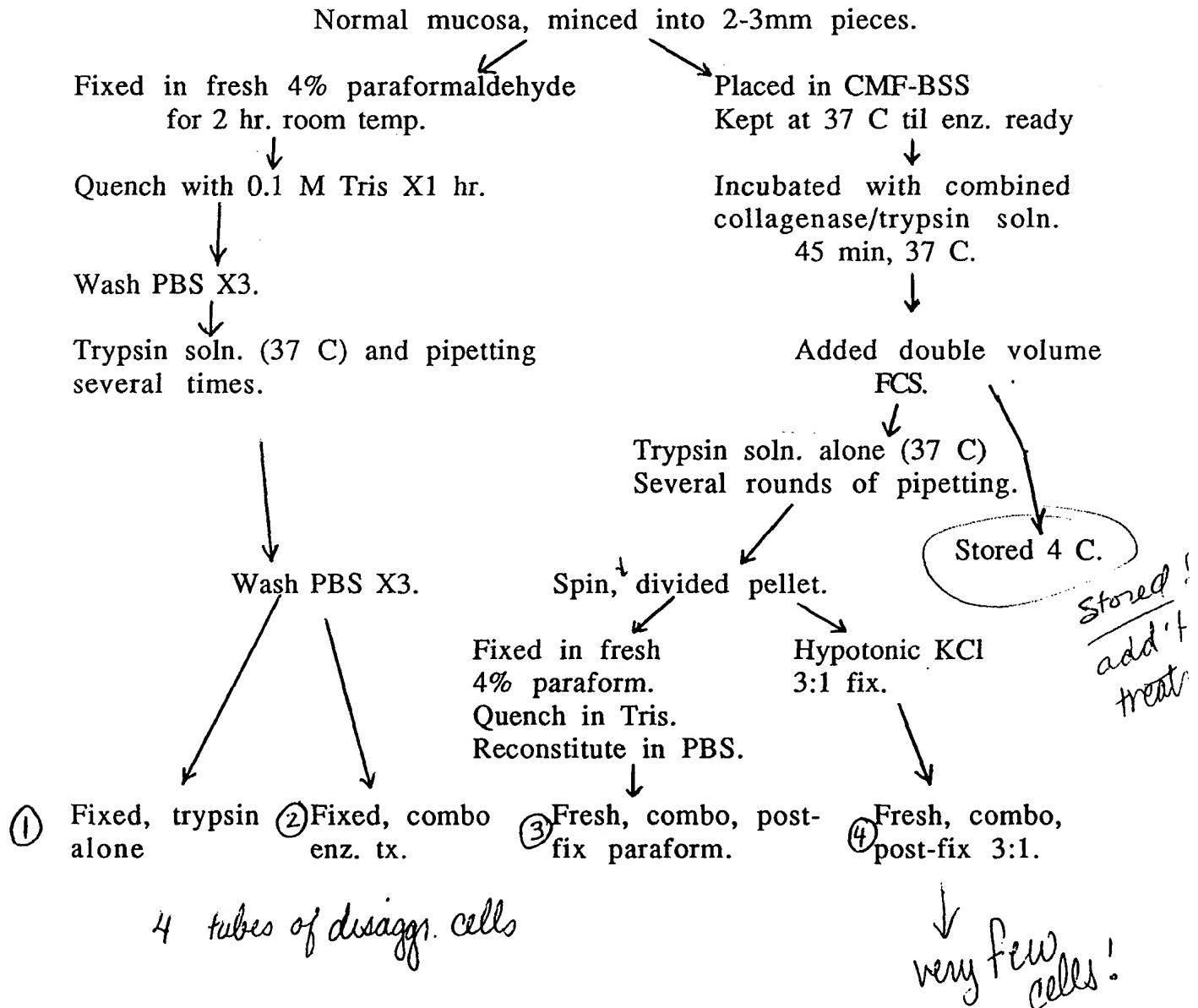
LY

- I. Detection steps for hybridization run.
- II. Cont. disaggregation protocol on fixed tissue.
- III. Order pepsin & materials for paraffin disagg.

III. Order supplies.

- *1. Sigma D5527 Dulbecco's PBS 500ml \$8.50 → 4
- * 2. Sigma H 8389 CMF-Hank's BSS 500ml \$9.25 → 2
- 3. Sigma: Trypsin inhibitor T 6522 100 mg. \$27.15 → 1
- 4. Sigma: Pepsin 1:60,000 P7102 5g \$49.30 → 1
- 5. Sigma Collagenase C 9407 100mg \$31.60 → 1
- 6. Sigma Cell dissociation kit CD-1 \$69.50 → 1

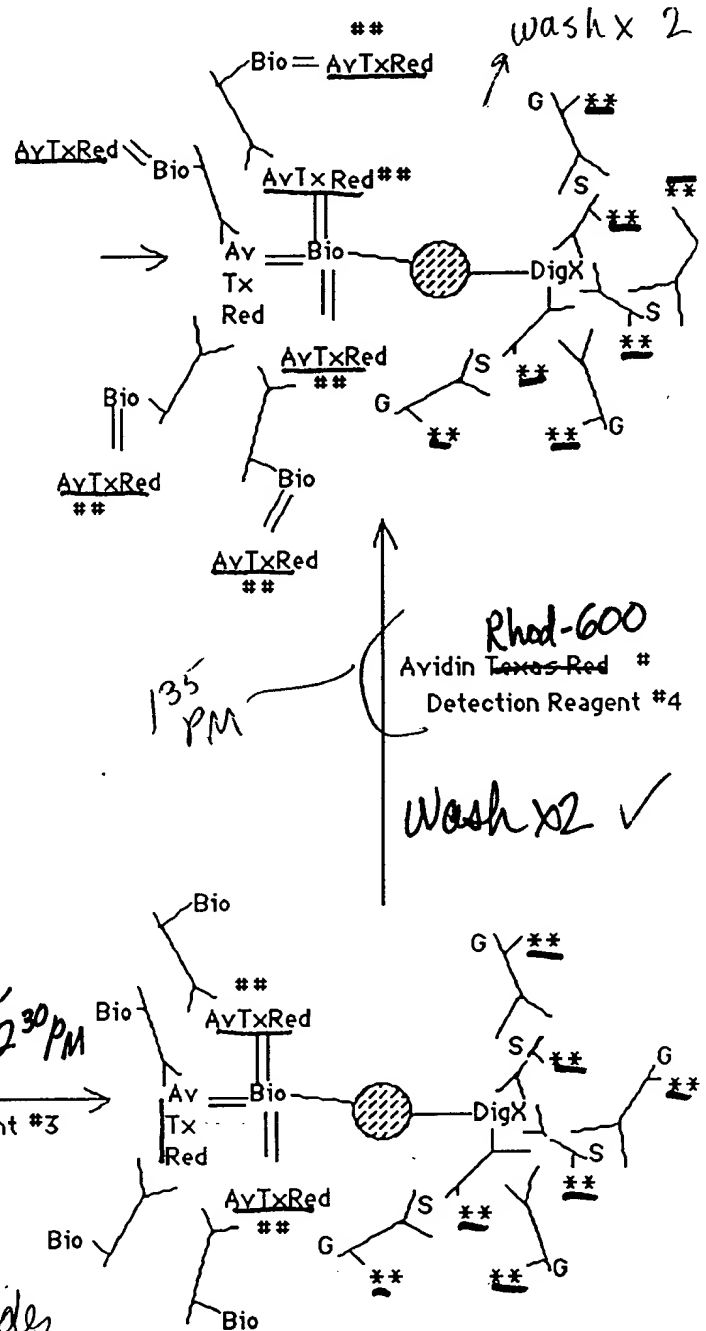
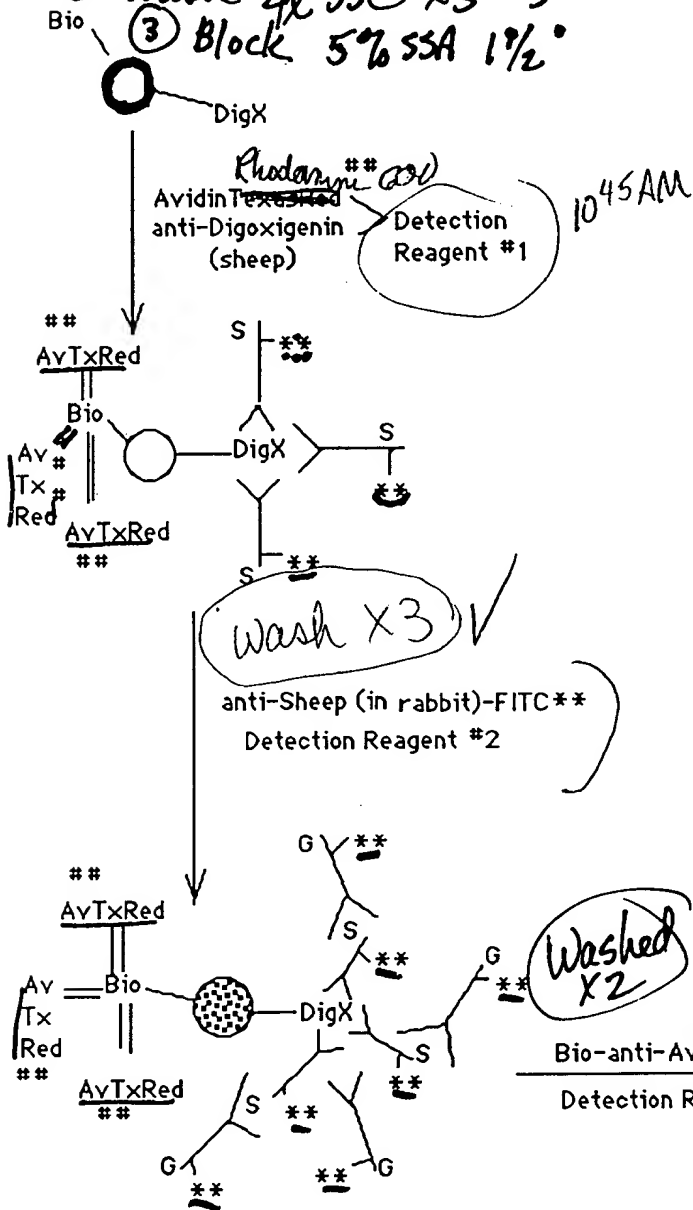
II. Summary of disaggregation steps:



In Situ Amplification Steps and Reagents

Yaremko

- ① Formamide 50% x 3 5'
- ② Wash 4x SSC x 3 3'
- ③ Block 5% SSA 1 1/2'



Made 3600 of each - for 16 slides

12000 SSA or RSA
2400 4xSSC/0.1% Triton
3600 of each fluorochrome

p amplification & washes, DAPI x 3-5'
Fast wash 4xSSC
DABCO & coverslip. Will examine in AM.

T:

- I. Evaluate colon disagg. samples.
- II. Hybridize colon sample if O.K.
- III. Collect more colon: Tumor + normal.
- IV. Evaluate slides from last run.

I. Colon samples:

A. Fixed, trypsin alone: good dispersion of cells, but still many clumps. Cytoplasm still largely intact. Nuclear detail good!

B. Fixed, combo collagenase/trypsin followed by trypsin: good dispersion of cell, better than trypsin alone. Cytoplasm often stripped from cells. Nuclear detail remains good.

C. Fresh, combo treatment, post-fixed in 3:1 fix: No cells.

D. Fresh, combo treatment, post-fixed paraformaldehyde: Cells destroyed, poor detail, poor staining, but disaggregated.

Conclusion: Fixed cells give better preservation with adequate dispersion. Will hybridize some from both the fixed preps to compare.

II. Hybridization: Eight slides to be done with MH5.15 ~~biotin~~. Dig X

1. Fixed trypsin alone with glue solution on slides.
2. ""
3. Fixed trypsin alone, no glue.
4. Fixed, combo enzyme treatment, with glue-treated slides
5. "" " "
6. Fixed combo treatment, no glue.
7. Touch prep, 90-11760, 95% alcohol fixed.
8. JK control normal lymphocytes.

MH5.15	Plac. DNA	ssDNA	KoAC	EtOH	Dextr/ Formam.
64	8	8	7	300	40/40

Combine, -70 X30 min, spin. Reconstitute, 5 min at 70 C. 37 C til use.

Slide prep:

- | | |
|---|---------------------------------|
| 1. Slide warmer 65 C X ³ 4 hr. | 6. Dehydrate & dry. |
| 2. RNase X1 hr, 37 C. | 7. Proteinase K 37 C 7.5 min. |
| 3. Wash 2X SSC X4 2 min. | 8. Dehydrate & dry. |
| 4. Dehydrate & dry. | 9. Apply probe & seal. |
| 5. 70% formd. 70 C X2min. | 10. 90 C x2 min, then 37 C o/n. |

III. Collect more colon: no reactions on schedule today